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PROSTASIN IS EXPRESSED IN BENIGN PROSTATIC HYPERPLASIA
AND REGULATES CELL PROLIFERATION AND INVASION VIA
iNOS, ICAM-1, AND CYCLIN D1

by

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B.S. Saint Louis University, 2006

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
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ABSTRACT

Prostasin is expressed in normal prostate epithelial cells but down-regulated in prostate cancers, while prostasin re-expression in invasive prostate cancer cells reduced invasion. We examined prostasin expression and function in benign prostatic hyperplasia (BPH). We evaluated prostasin expression in 12 BPH specimens by immunohistochemistry, and evaluated the impact of prostasin silencing by siRNA on the expression of the inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), and cyclin D1, as well as on cell proliferation and invasion, using the BPH-1 human prostate epithelial cell line model. Prostasin expression was localized in the glands of BPH tissues by immunohistochemistry, in either the tall columnar-shaped or the flattened epithelial cells. We silenced prostasin expression by >50% at both the mRNA and protein levels using siRNA in the BPH-1 human prostate epithelial cell line, and this silencing of prostasin expression was associated with an induction of iNOS and ICAM-1 expression and a down-regulation of cyclin D1 expression. The protein expression of EGFR, a putative prostasin substrate, was not affected by prostasin silencing in this cell line. The prostasin-silenced cells displayed a reduced cell proliferation rate and reduced invasiveness, cell behaviors regulated by cyclin D1, iNOS, and ICAM-1 in the BPH-1 cells. We believe that this down-regulation of cyclin D1 is due to prostasin's augmentative effect on iNOS. We also believe that the decrease in cell motility is due to an increase in iNOS and ICAM-1 as well as a decrease in cyclin D1, since all of these molecules can play a role in cell motility. In conclusion, Prostasin is somehow involved in the regulation of inflammatory gene expression (iNOS and ICAM-1) in prostate epithelial cells, as well as cyclin D1 expression, cell proliferation and

invasion, involving molecular mechanisms different than those in the prostate cancer cells. These studies suggest that prostaticin is a player in the glandular components of benign prostatic hyperplasia.

This thesis is dedicated to my parents, Bruce and Kathy Hatfield, without whom my educational and life experiences would have never been possible.

ACKNOWLEDGMENTS

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INTRODUCTION

The prostate is a male-specific sex accessory organ located at the base of the bladder [1]. The prostate does not have a clearly defined physiological function [1]. It is thought that the prostate produces somewhere around 20-30% of the ejaculate fluid. It may also produce the proteolytic enzymes, acid phosphatase, prostate-specific antigen, and zinc that function in sperm motility and penetration, but these molecules are not essential for fertility [1]. The prostate has three anatomically distinct zones as described by McNeal, the central zone, the peripheral zone, and the transitional zone [1]. The transition zone surrounds the prostatic urethra and comprises 5% of the glandular tissue [1]. The next region is the central zone. The central zone surrounds the ejaculatory ducts [1]. Another region is called the peripheral zone [1]. It was historically thought that BPH only arises from the transition zone, however, new evidence is showing that the volume of the central/peripheral zone increases from the normal volume of 30cm³ [2]. Since it is rare to see BPH nodules arising from the central/peripheral zone, it is thought that the nodules developing in the transition zone are protruding into the peripheral/central zone and causing it to increase in volume during BPH progression [2]. A fibromuscular stroma surrounds the zones of the prostate that serves as the bed, or non-functional framework of the prostate [1].

Prostate, the namesake of the glycosylphosphatidylinositol (GPI)-anchored extracellular serine protease prostatic serine protease (PRSS8)/CAP-1, is affected by two major diseases, prostate cancer and benign prostatic hyperplasia (BPH). Benign prostatic hyperplasia is a very common condition among men, especially older men [3]. Age and family history are the most common risk factors [1]. Eighty-four percent of men over the age of 70 are

living with this condition [4]. BPH is essentially an enlarged prostate that causes LUTS, lower urinary tract symptoms. This can include BOO (bladder outlet obstruction), weak stream, hesitancy, frequent urination, and acute urinary retention [5]. At the histological level BPH is hyperplasia of the stromal and glandular components of the prostate [5]. When the prostate enlarges, instead of pushing outward into the body cavity, it actually expands inward and cuts off the flow of urine through the urethra [6].

It is the current opinion in the field that there are four possible causes of BPH. The first is hormone-mediated. Steroid hormones play a significant role in the growth and function of the normal prostate as well as the development of BPH [7]. Intraprostatic estradiol levels increase in men with age along with prostate volume [7]. Later in life men start to have more estrogen than androgen [7]. This is due to the increase in aromatase, which aromatizes androgens into estrogen [7]. It is thought that this increase in the ratio of estrogen to testosterone could be the reason for the enlarged prostate [7]. Another theory is the stem cell theory, which postulates the development of BPH through an increase in the number of stem cells or through an abnormal increase in clonal expansion of amplifying or transit cells [8]. There is a third theory called “embryonic reawakening” [9]. This is the idea that ontogenic processes occur later in life and result in the formation of BPH nodules [9]. In 1937 it was proposed that inflammation of the prostate was the cause of BPH [10]. Since then these other theories have taken off as the accepted cause of BPH [10]. However, there is now a reversion back to the original idea that BPH is an inflammatory disease[10].

Currently there are minimally invasive therapies available for BPH, including microwave thermotherapy, water-induced thermotherapy, interstitial device-based

therapy, transurethral needle ablation, and transurethral resection of the prostate [11]. There are three classes of drug treatment which include 5 alpha reductase inhibitors, alpha-blockers, and anti-inflammatories. Dutasteride (Avodart) and Finasteride (Proscar) are 5-alpha-reductase inhibitors, and drugs such as Tamulosin are all alpha-blockers. The 5-alpha-reductase inhibitors work to inhibit the production of the hormone dihydrotestosterone (DHT), a hormone that may be responsible for prostate enlargement [12]. A decrease in DHT can result in a promotion of prostate involution and a decrease in angiogenesis [12]. Alpha-blockers work on peripheral arteries and veins to inhibit vasodilation and smooth muscle contraction, relieving some of the symptoms of BPH [13]. Anti-inflammatories work to decrease inflammation that is thought to cause BPH.

Prostasin is a GPI-anchored serine protease[14]. The expression and functional role of prostasin in the prostate with regard to prostate cancer have been extensively studied. For BPH, however, the information on prostasin expression and function is lacking. Prostasin is expressed abundantly in normal prostate epithelial cells but is down-regulated in high-grade prostate cancers or those associated with the hormone-refractory phenotype, due to promoter DNA hypermethylation and increased expression of transcription repressors [15-18]. Prostasin re-expression in highly invasive human prostate cancer cell lines reduced invasiveness possibly as a result of epidermal growth factor receptor (EGFR) signal modulation via protease-dependent and independent mechanisms [19,20]. Currently the only well known functions of prostasin are its activation of the epithelial sodium channel γ -subunit, its maintenance of epidermal integrity, and its attenuation of inflammation-induced gene expression [21].

We examined pathologically confirmed BPH tissues presenting both stromal and glandular hyperplasia for prostasin expression by immunohistochemistry, and localized prostasin protein expression in two distinct types of glandular epithelial cells. We also demonstrated prostasin expression in an immortalized but non-transformed human prostate epithelial cell line, the BPH-1 cell line [21]. We investigated prostasin's functional role in the BPH-1 cell line by silencing prostasin expression using an siRNA. In association with prostasin silencing, iNOS, ICAM-1/CD54 expression was induced at the mRNA and protein levels, while the expression of cyclin D1 at the mRNA and the protein levels was reduced. Prostasin-silenced BPH-1 cells were shown to proliferate at a lower rate and were less invasive.

HYPOTHESIS

Prostasin is known to be required for terminal epithelial differentiation [22]. It also includes data from a previous paper that prostasin's down-regulation could be due to inflammation [23]. I hypothesize that prostasin silencing in the BPH-1 cell line will change the expression levels of iNOS, ICAM-1, and Cyclin D1, therefore changing the proliferation rate and invasiveness of the cell line.

MATERIALS AND METHODS

Tissue preparation and immunohistochemistry:

Paraffin-embedded prostate tissues from patients undergoing transurethral resection of the prostate (TURP) for BPH were obtained from Florida Hospital in Orlando with informed consent and institutional approval. Twelve specimens with pathologically confirmed presentation of BPH were used for this study. The procedures for immunohistochemistry were essentially as described before [15]. Sections 4 μ m thick were deparaffinized, rehydrated, and subjected to antigen retrieval in citrate acid buffer (pH 6.0). The prostate sections were treated with 3% H₂O₂ in methanol followed by blocking in 5% goat serum in 1X PBS (pH 7.2). Then sections were then incubated with a rabbit anti-human prostatic polyclonal antibody [24] in the blocking buffer at 4°C overnight. Other primary antibodies used include: rabbit anti-human androgen receptor antibody (1:50), rabbit anti-human cMet antibody (1:50), rabbit anti-human PSA antibody (1:200). The sections were washed with 1X PBS for 3 times at 5 minutes each following the primary antibody incubation; before incubation with either a goat anti-rabbit IgG serum (Vector Laboratories, Burlingame, CA, 1:100) or a goat anti-mouse IgG serum (Vector Laboratories, Burlingame, CA, 1:100). The sections were then washed with 1X PBS for 3 times at 5 minutes each. Then they were incubated with PAP (goat or mouse) (1:200) in 5% goat serum for one hour. The sections were then washed again in 1X PBS for 3 times at 5 minutes each. The color reaction was performed by incubation the sections with DAB (3,3'-diaminobenzidine tetrahydrochloride) solution (0.5 mg/ml in 0.03% H₂O₂) for 15 minutes. The sections were counterstained with hematoxylin and photographed.

Cell Culture:

BPH-1, an immortalized but non-transformed benign prostatic hyperplasia cell line was kindly provided by Dr. Simon Hayward of the Vanderbilt University Medical Center (Nashville, TN), and was cultured in RPMI-1640 supplemented with 5% fetal bovine serum (FBS) in a humidified incubator at 37°C with 5% CO₂ and 95% air. Passaging was performed as described [25].

siRNA Transfection of BPH-1 cell line:

The prostatin-specific short interfering RNA (siRNA) and a randomized control siRNA were described previously (Tong et al., 2004) and were synthesized by Integrated DNA Technologies (Coralville, IA). BPH-1 cells were seeded in a 6-well plate at 3×10^5 cells per well and were transfected with 125 picomoles of siRNA per well using Lipofectamine-2000 (Invitrogen, Carlsbad, CA). The siRNA and Lipofectamine were allowed to incubate together for 15 minutes before being in contact with the cells. After 15 minutes, the solution was then added to each well containing 0.8ml of fresh media. Five hours after transfection, the cells were given fresh media. Forty-eight hours after transfection, the cells were collected for RNA isolation, lysed in RIPA buffer for western blot analysis, or trypsinized for proliferation and invasion assays.

Western Blot Analysis:

The procedures for western blot analysis were as described previously [15]. Briefly, cells were washed two times in 1X PBS then lysed in RIPA buffer at 4°C for 15

minutes (RIPA buffer = 20mM Tris pH8.0 containing 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM PMSF, 2 /ml aprotinin, 2ug/ml leupeptin, 50ug/ml soybean trypsin inhibitor, 10mM NaF, 1mM Na₃VO₄). The mixture was then centrifuged at 12,000 rpm for 10 minutes in 4°C. The supernatant was then transferred to a new tube and the protein concentration was determined by Dc protein assay (Bio-Rad). For iNOS western blots, cells were lysed directly in 1% SDS (in 25 mM Tris-HCl, pH 7.6, 140 mM NaCl, and 5 mM EDTA), and boiled for 5 minutes. Equal amounts of total protein for each sample were resolved on SDS-PAGE gel and electro-transferred to a nitrocellulose membrane. Nitrocellulose membranes were blocked in either 5% milk in T-BST or 1% BSA in TBS-T (20mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween-20), and incubated with the appropriate antibodies. The antibodies used were human prostasin ([24], used at 1:4,000), EGFR (sc-03, Santa Cruz Biotechnology, Santa Cruz, CA; 1:4,000), iNOS (610328), CD54/ICAM-1 (611704) and cyclin D1 (554181) (BD Biosciences, San Jose, CA; 1:800, 1:500 and 1:1,000), and β -tubulin (Sigma-Aldrich; 1:4,000). After incubation with the appropriate primary antibody, the membranes were washed and incubated for one hour with the corresponding secondary antibody conjugated to the horseradish peroxidase (HRP) (1:10000). The membranes were then washed and subjected to enhanced-chemiluminescence reaction (ECL, Pierce Biotechnology, Inc., Rockford, IL) before exposure to X-ray films.

RNA Isolation and Analysis by Reverse Transcription and Real-time Polymerase Chain Reaction:

Cells were lysed with 500ul of TRIZOL Reagent (Invitrogen) and RNA was isolated according to manufacture's protocol. RNA concentrations were determined using spectrophotometry. Reverse transcription was carried out using the iScript cDNA Synthesis Kit (Bio-Rad, Herules, CA). Real-time PCR was carried out on a Bio-Rad MyiQ system using the iQ SYBR Green Supermix reagents (Bio-Rad). PCR program and methods of quantification were described previously [18]. PCR primers' sequences were designed with the aid of the Beacon Designer 4.0 software (PREMIER BioSoft International, Palo, Alto, CA) and synthesized by Integrated DAN Technologies (Coralville, IA). The primers' sequences are listed as below, in the order of forward and reverse:

B-Actin: 5'-TGG ACA TCC GCA AAG ACC TG-3', 5'-CCG ATC CAC ACG GAG TAC TT-3'.

Prostasin: 5'-ATC TTG GAT TAC TCC GGT CGG-3', 5'-ACA CAT GGA CGC CTT CAT AGG-3'.

Cyclin D1: 5'-GAC CTT CGT TGC CCT CTG-3', 5'-AGG CGG TAG TAG GAC AGG-3'

iNOS: 5'-ATC TCT GGT CAA GCT GGA TGC-3', 5'-GCC TTA TGG TGA AGT GTG TCT TG-3'

ICAM-1: 5'-ACA GTC ACC TAT GGC AAC-3', 5'-TGG CTT CGT CAG AAT CAC-3'

Proliferation assay:

The siRNA-transfected BPH-1 cells were trypsinized and seeded in a 96-well plate at a density of 7.5×10^3 per well in triplicates for the CyQUANT[®] NF Cell Proliferation Assay using a kit (Invitrogen). At 3 hours after seeding, cells were treated with the CyQUANT reagent for 30 minutes. The fluorescence readout was measured using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Inc., Walnut Creek, CA). This time point was designated as Day 0. At 24, 48, and 72 hours after cell seeding, the procedures were repeated and the time points were designated as Day 1, Day 2, and Day 3.

Invasion assays:

The procedures were modified from those described previously [15], using Transwell invasion chambers with 8- μ m pore-size polycarbonate filters (Corning Inc., Lowell, MA). The insert-filters were pre-coated with Matrigel (Sigma-Aldrich), at 50 μ g/20 μ l per insert. BPH-1 cells transfected with siRNA were trypsinized and seeded in the Transwell inserts at 1×10^5 cells per well. The cells were allowed to invade for 24 hours. The inserts were then washed with cold 1x PBS twice and fixed with 4% paraformaldehyde for 30 minutes, washed again with 1x PBS and stained with 0.2% crystal violet. The cells remaining on the top of the filters were removed with a cotton tip, and the cells on the underside of the filters were photographed. The experiment was repeated three times.

Statistical analysis:

Results of western blotting were digitally scanned and quantitatively analyzed by densitometry. Data of western blotting were normalized with the value of β -tubulin. Gene expression evaluation using the quantitative real-time PCR data was performed by comparing the means of molecular equivalence of the target gene to the β -actin message. Statistical difference was evaluated by the Student t-Test (one-tailed, equal variance) and $p < 0.05$ was used to reject the null hypothesis.

RESULTS

Prostasin expression in BPH tissue and the BPH-1 cell line

We evaluated 12 pathologically confirmed BPH tissues for prostasin expression in the epithelial cells. We have observed two distinct types of the glandular epithelial cells, a tall columnar-shaped and a flattened. In Figure 1A, prostasin protein expression in the tall columnar-shaped glandular epithelial cells is shown, this staining pattern was observed in 11 out of the 12 tissues. In the same view field, there are also areas with the same cell type but are negative for prostasin staining, which were observed in 10 out of the 12 tissues. In Figure 1B, the basally located nuclei of the tall columnar-shaped glandular epithelial cells can be clearly seen, typical of the secretory luminal cells. In Figures 1C and 1D, prostasin protein expression in the flattened glandular epithelial cells is shown, this staining pattern was observed in 11 out of the 12 tissues. We have also identified areas of flattened glandular epithelial cells with negative prostasin staining in 9 out of the 12 tissues (not shown). Overall, in 8 out of the 12 tissues, we found all four different staining patterns, i.e., columnar/prostasin-plus, columnar/prostasin-minus, flattened/prostasin-plus, and flattened/prostasin-minus. It is unclear what the two distinct types of glandular epithelial cells represent in terms of BPH pathology or cell biology. However, it is important to point out that some of the areas of tissue were negative, meaning they are not ‘normal’. An absolutely normal prostate would have 100% of the epithelial cells stained for prostasin.

Prostasin is also expressed in the BPH-1 cell line, as shown by the western blot results in Fig. 2. We then sought to investigate prostasin’s functional role in BPH by down-regulating its expression in the BPH-1 cell line. In Fig. 2, we show the effect of a

prostasin-specific siRNA (Pro-siRNA) transfected in the BPH-1 cells on prostasin protein expression, which was reduced by 55% ($p < 0.05$, densitometry data not shown), when compared to the untreated cells (Parent), or cells treated with a randomized control siRNA (Con-siRNA) or Lipofectamine 2000 alone (Lipids). The prostasin mRNA expression in the Pro-siRNA treated cells was also reduced, by 68% when compared to the untreated Parent cells (Table I). The liposomes or the control siRNA did not have an effect on prostasin mRNA (Table I) or protein expression (Fig. 2, $p > 0.05$, among the controls).

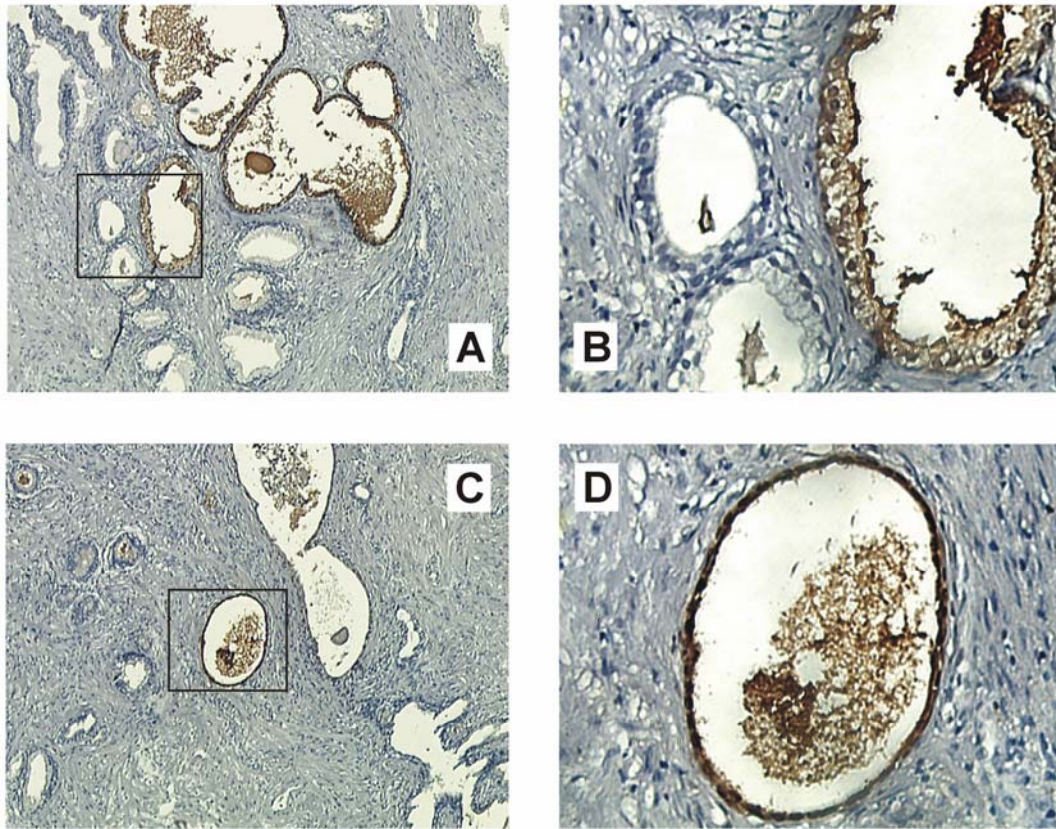


Figure 1 Expression of prostatic stromal protein in BPH. BPH tissues were subjected to immunohistochemistry analysis. Prostatic stromal protein-positive staining is shown by the brown color.

A. A representative area of glandular BPH with tall columnar-shaped epithelial cells stained for prostatic stromal protein expression. This area also shows the same cell type negative for prostatic stromal protein expression. Image was taken with a 10x objective lens and a 0.45x coupler to a SONY DXC-950 3CCD camera. **B.** A high-magnification view of the boxed area in **A** (40x objective lens). **C.** A representative area of glandular BPH with the flattened epithelial cells stained for prostatic stromal protein expression. Magnification: same as in **A**. **D.** A high-magnification view of the boxed area in **C** (40x objective lens).

Table 1. Analysis of Immunohistochemistry

BPH IHC for Prss8	Columnar		Flat	
Slide Numbers	Positive	Negative	Positive	Negative
1	X	X	X	X
2	X	X	X	X
3	NE	NE	X	X
4	X	X	X	X
5	X	X	X	X
6	X	X	X	X
7	X	NE	NE	NE
8	X	X	X	X
9	X	X	X	NE
10	X	X	X	X
11	X	X	X	NE
12	X	X	X	X
	11/12	10/12	11/12	9/12

Prostasin Silencing in BPH-1 Cells Increased iNOS and ICAM-1 Expression

Prostasin expression is repressed during inflammation while a forced prostasin expression could attenuate inflammatory gene expression response, specifically, that of the inducible nitric oxide synthase (iNOS) [19,23]. We analyzed the prostasin-silenced BPH-1 cells (Pro-siRNA) for changes of iNOS expression, and found that at the mRNA level, there was an induction of iNOS expression to > 13 fold when compared to the untreated parent cells (Table I). The liposomes or the control siRNA did not have an effect on iNOS mRNA expression. At the protein level, only the prostasin-silenced BPH-1 cells were shown to express a detectable amount of iNOS (Fig. 2).

In the classic human prostate cancer cell lines, two that do not express prostasin (DU-145 and PC-3) [15] expressed a high level of intercellular adhesion molecule-1 (ICAM-1) [26], while one with prostasin expression (LNCaP) [15] did not express ICAM-1 [26]. ICAM-1 expression in the DU-145 and PC-3 cells responded to cytokine stimulation [27], conditions reminiscent of inflammation, which represses prostasin expression. We asked if prostasin silencing in the BPH-1 cells could affect ICAM-1 expression because prostasin was proposed to regulate cytokine-induced inflammatory gene expression [23]. An induction of ICAM-1 expression to 2.47 fold at the mRNA level (Table I), and to ~2.7 fold at the protein level (Fig. 2, $p < 0.05$, versus the controls), was observed in the prostasin-silenced BPH-1 cells, when compared to the untreated cells. The liposomes or the control siRNA did not have an effect on ICAM-1 mRNA (Table I) or protein expression (Fig. 2, $p > 0.05$, among the controls).

Prostasin silencing in BPH-1 cells down-regulated cyclin D1

Prostasin has recently been shown to modulate EGFR-mediated cell signaling in prostate cancer cells by protease-dependent and protease-independent mechanisms [19,20]. EGFR-mediated cell signaling regulates cyclin D1 expression and cellular responses mediated by cyclin D1 in the prostate [26]. We have observed both prostasin-positive and prostasin-negative glandular epithelial cells in the clinical BPH tissues, and we sought to investigate prostasin's functional role in this context by down-regulating its expression in the BPH-1 cell line. In Figure 2, we show the effect of a prostasin-specific siRNA (Pro-siRNA) transfected in the BPH-1 cells on prostasin protein expression, which was reduced by $> 50\%$ ($p < 0.05$, densitometry data not shown), when compared to the untreated cells (Parent), or cells treated with a randomized siRNA (Con-siRNA) or lipids alone (Lipids). The prostasin protein expression levels were not different among the three control cell types ($p > 0.05$). The prostasin mRNA expression in the Pro-siRNA treated cells was also reduced by $> 50\%$ ($p < 0.05$) when compared to the control cells. The protein expression of cyclin D1 of the Pro-siRNA treated BPH-1 cells was reduced by $> 50\%$ as determined by the western blots ($p < 0.05$, versus the controls). The three control cell types were shown to express similar levels of cyclin D1 ($p > 0.05$). The cyclin D1 down-regulation associated with prostasin silencing is at the transcriptional levels as the cyclin D1 mRNA in the Pro-siRNA treated cells was down-regulated by $> 50\%$ as well ($p < 0.05$, versus the controls). For prostasin as an extracellular serine protease to regulate cyclin D1 expression at the transcriptional level, a transmembrane molecular mechanism must serve as a relay for the signal. We examined the protein expression of EGFR, a putative prostasin substrate and a regulator of cyclin D1

expression. EGFR protein expression, however, was not affected in association with prostatic silencing in the BPH-1 cells, as shown in Figure 2. Prostatic modulation of EGFR-mediated signaling is accompanied by EGFR cleavage in the extracellular domain [20], or changes of EGFR protein expression [19]. In the BPH-1 cells, we did not observe these trademarks for the previously known mechanisms of EGFR signal modulation by prostatic. A novel molecular mechanism, different from that mediated by EGFR, must be suggested for prostatic's regulatory role in cyclin D1 expression.

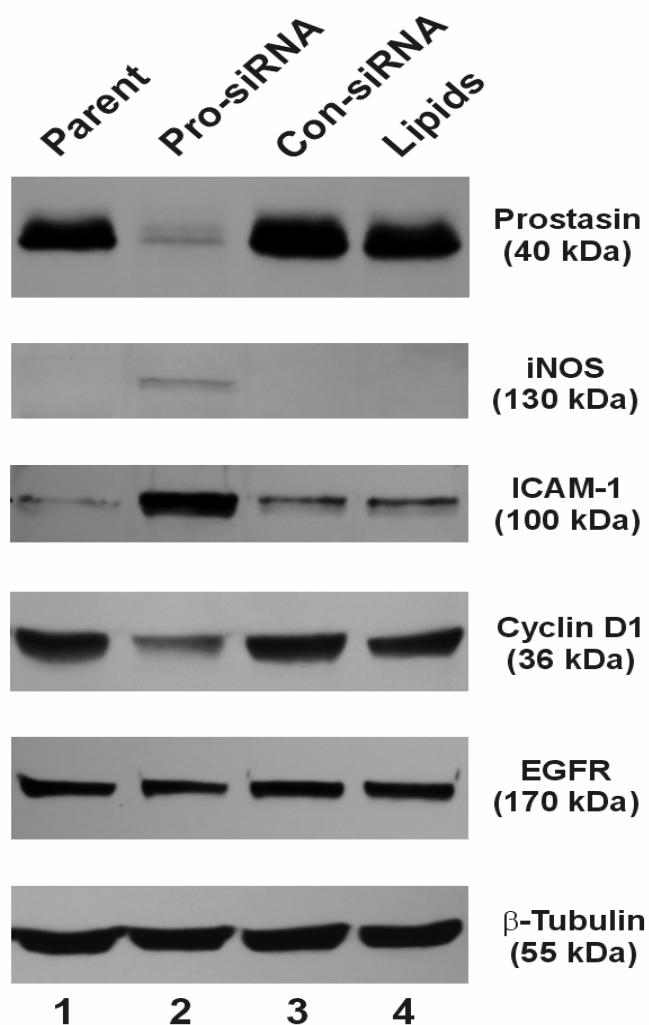
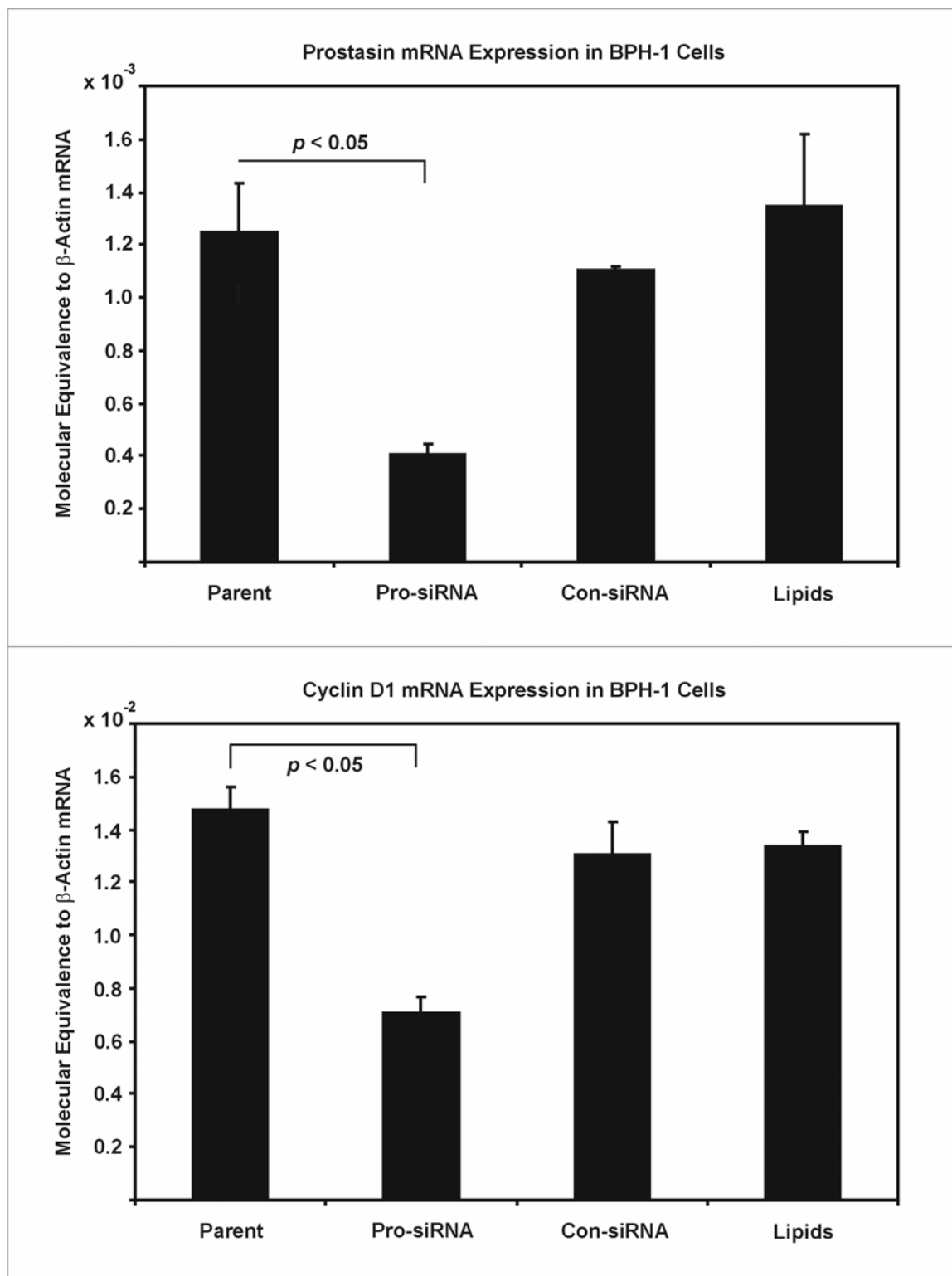
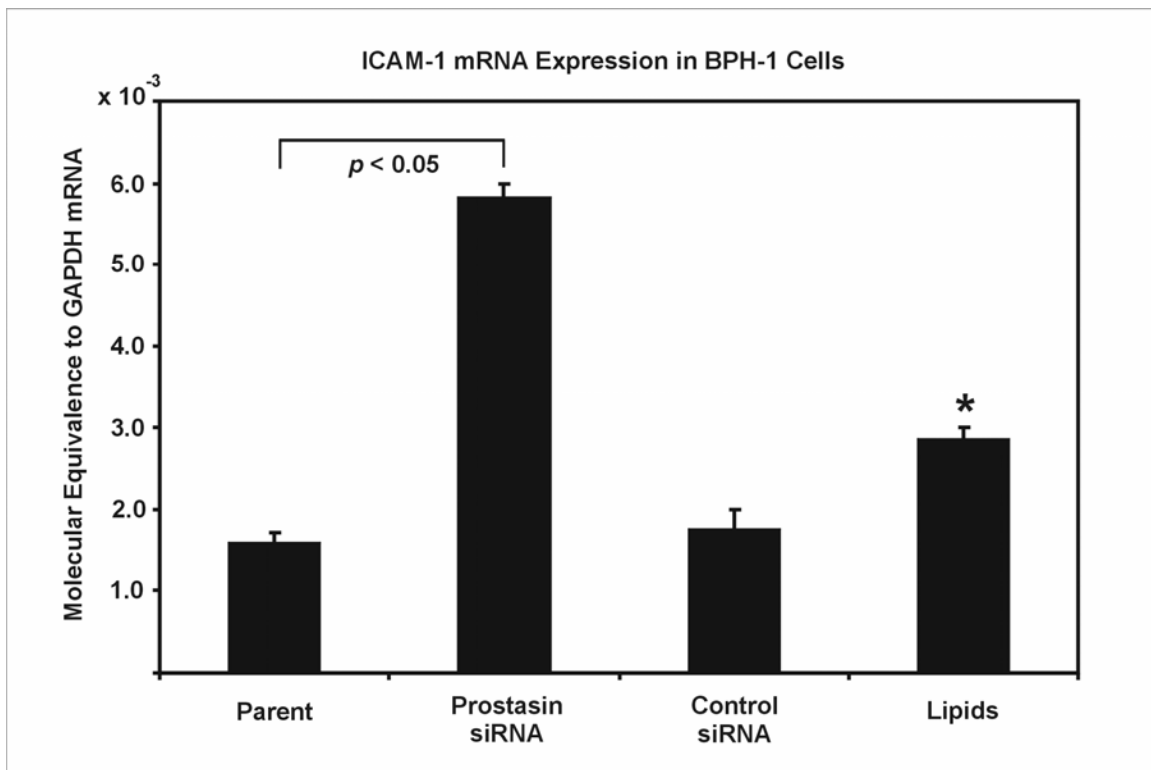
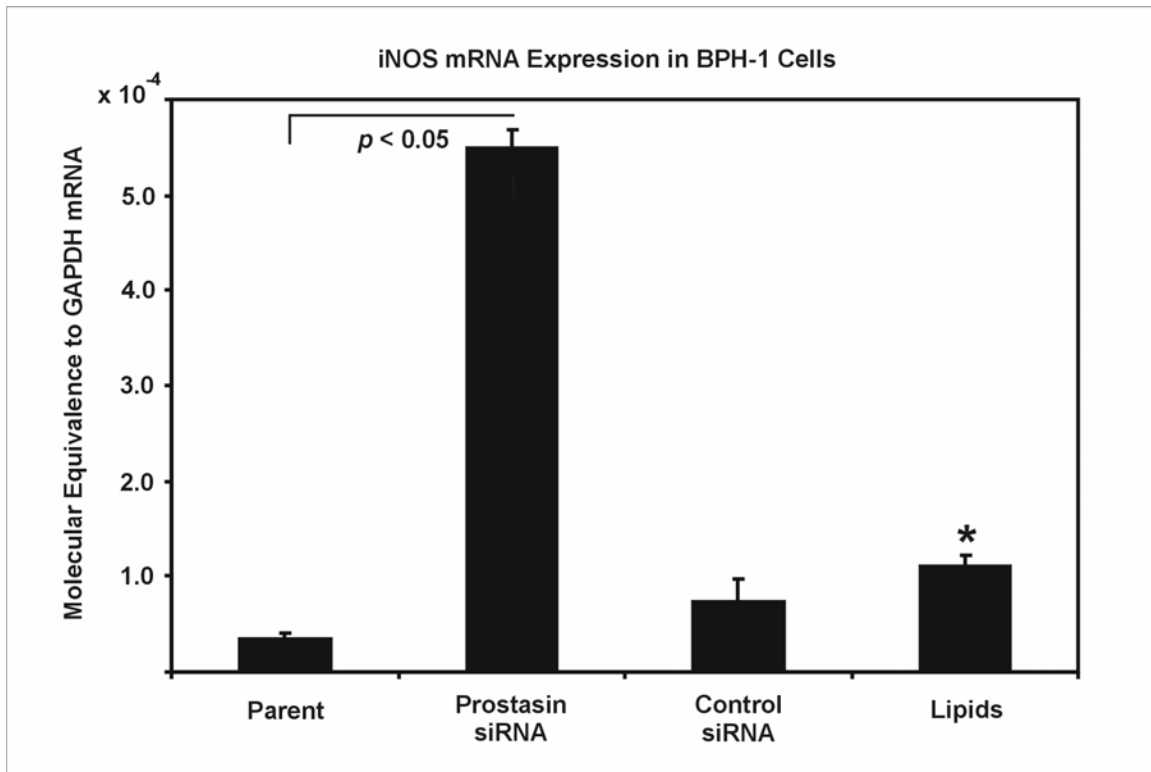


Fig. 2. Prostasin silencing in BPH-1 cells resulted in protein expression changes of iNOS, ICAM-1, and cyclin D1. Images are representative of three independent western blotting experiments. Samples loaded in the gels are indicated in the figure: **Parent**, untreated BPH-1 cells; **Pro-siRNA**, cells transfected with a prostatin-specific siRNA; **Con-siRNA**, cells transfected with a randomized control siRNA; **Lipids**, cells treated with Lipofectamine 2000 alone. For the **Prostasin** blots, 10 µg of total protein per sample were analyzed by non-reducing SDS-PAGE. For the **iNOS** blots, 40 µg of total protein per sample were analyzed by reducing SDS-PAGE. For the **ICAM-1**, **Cyclin D1**, **EGFR**, and **β-Tubulin** blots, 45 µg of total protein per sample were analyzed by reducing SDS-PAGE.





Prostasin silencing in BPH-1 cells decreased cell proliferation

It has recently been shown that over-expression of cyclin D1 in the BPH-1 cell line resulted in increased cell proliferation in vitro, and in larger as well as more vascularized grafts when recombined with rat urogenital mesenchyme (rUGM) under the kidney capsule of severe combined immunodeficient (SCID) mice [25]. We asked if the cyclin D1 down-regulation associated with prostasin silencing in the BPH-1 cells would have an impact on cell proliferation. In Figure 3, we show that the Pro-siRNA treated cells were proliferating at a rate which was only at ~ 60% of the cells treated with Con-siRNA or Lipids on Day 3 ($p < 0.05$). The cells treated with Con-siRNA or Lipids were also proliferating at lower rates (~ 74% on Day 3) than the untreated BPH-1 (Parent) cells ($p < 0.05$). We attribute the reduced proliferation rates of the Con-siRNA or Lipids treated cells to the effects of the liposomes as all cell types except the parent were treated with an equal amount of liposomes. It has been reported previously that liposomes alone could produce growth inhibitory effects on cultured cells [27]. The liposome-induced growth suppression did not mask the effect of prostasin silencing on proliferation because the proliferation rate of the Pro-siRNA treated cells was lower than that of the cells treated with either the Con-siRNA or Lipids. The liposomes, however, did not have a repressive effect on prostasin expression (Figure 2). The growth inhibition effect secondary to prostasin silencing is hereby viewed to be independent of the liposomes' effects and specific to the prostasin-silencing event.

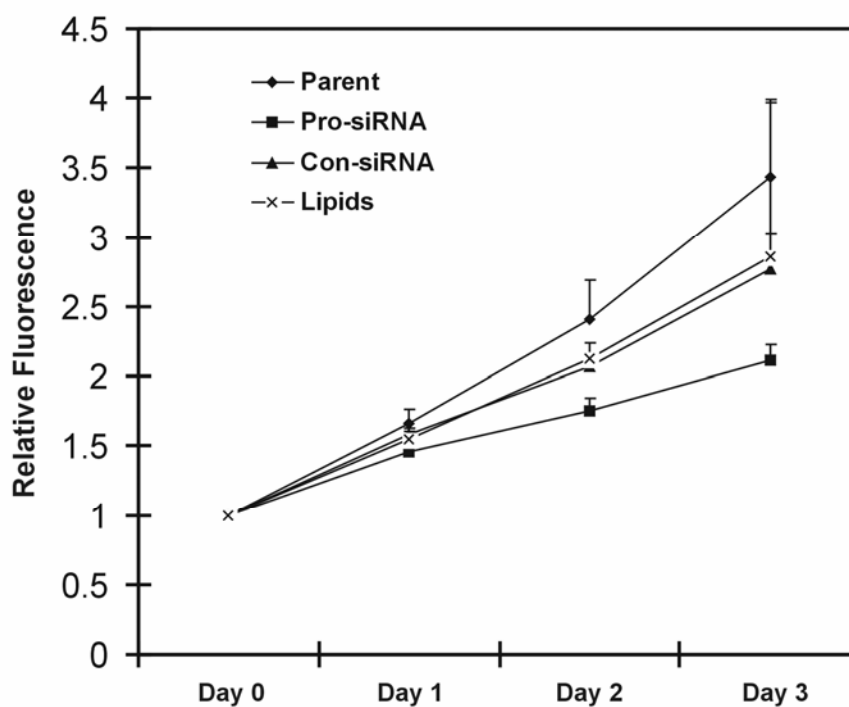


Figure 3 Prostaticin silencing in BPH-1 cells decreased cell proliferation. The siRNA-transfected BPH-1 cells were subjected to the CyQUANT® NF Cell Proliferation Assay along with the controls, as indicated in the figure. At times indicated, the fluorescence was measured as a surrogate of cell density. The data were expressed as the relative increases from the first point (Day 0), and were from three independent experiments.

Prostasin silencing in BPH-1 cells decreased cell invasion through Matrigel

Over-expression of cyclin D1 in the BPH-1 cells also induced cell migration changes that resulted in increased invasion through Matrigel [15]. We asked if the cyclin D1 down-regulation associated with prostasin silencing in the BPH-1 cells would impact invasion. In Figure 4, we show that the Pro-siRNA treated BPH-1 cells were no longer invading through the Matrigel. We performed the experiments as described by He et al. [25], using exactly the same amount of Matrigel coating and the same number of starting cells (100,000), except that we had allowed the invasion to proceed for twice as long (24 hours versus 12 hours). Even under the prolonged invasion, Pro-siRNA treated BPH-1 cells were not invasive. The data presented in Figure 4 are therefore, qualitative but not quantitative because the Pro-siRNA treated cells had hardly any invasion. The differences of invasion we had observed between the Pro-siRNA treated cells and the control cells were not the result of a differential proliferation rate, because at the time point of stopping the invasion assays (i.e., 24 hours, equivalent to Day 1 in the proliferation assays), the differently treated cells did not show a measurable proliferation rate difference. We evaluated the proteolytic profiles of matrix metalloproteases (MMP-2 and MMP-9) by gelatin zymography assays of the secreted MMP's in the culture medium, no difference was observed for the differently treated cells (data not shown). The reduced invasion of the Pro-siRNA treated BPH-1 cells is most likely a change of migratory potential, which can be directly regulated by cyclin D1 [25]. Once again, the liposomes produced an inhibitory effect on cell invasion as cells treated with the Con-siRNA or Lipids were equally less invasive than the untreated (Parent) cells. Cationic

liposomes used for transfecting nucleic acids into cultured cells, such as the Lipofectamine 2000 reagent used in this study, were also shown previously to inhibit tumor cell metastatic capabilities [28]. But the Pro-siRNA treated cells were non-invasive, clearly different from the cells treated with the Con-siRNA or Lipids. The invasion inhibition effect secondary to prostatic silencing is also viewed to be independent of the liposomes' effects and specific to the prostatic-silencing event.

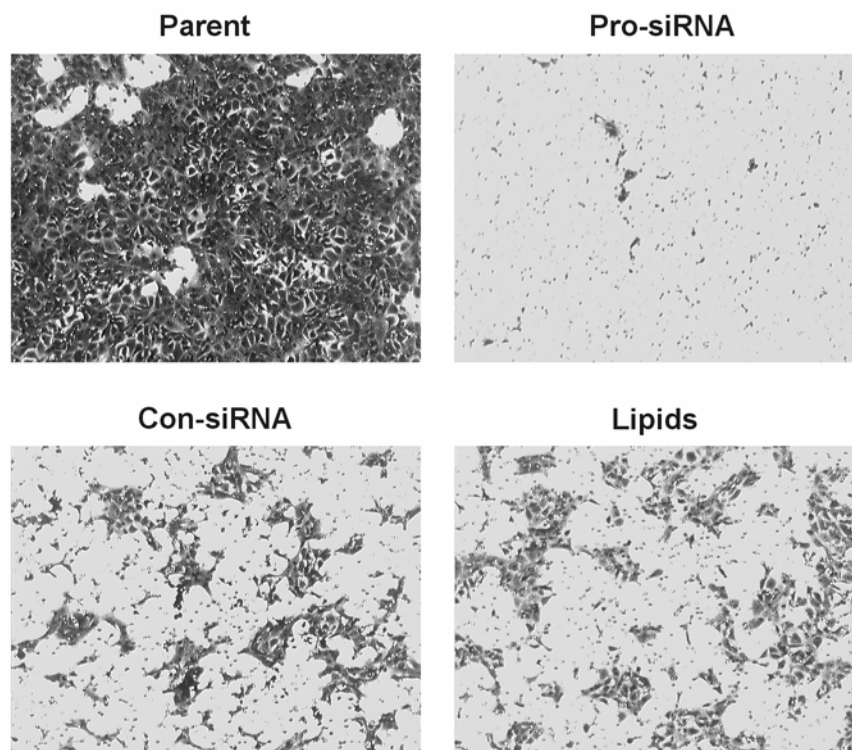


Figure 4 **Prostasin silencing in BPH-1 cells decreased cell invasion through Matrigel.** The siRNA-transfected BPH-1 cells were subjected to Matrigel invasion assays along with the controls, as indicated in the figure. Images shown were representative of three independent experiments

DISCUSSION

In this study, we have demonstrated prostatic expression in the glandular epithelial cells in prostate tissues presenting clinical BPH, and at the same time shown areas where the glandular epithelial cells are negative for prostatic expression. At present, the pathological and molecular mechanisms for this differential expression of prostatic in BPH tissues are not known. As the majority of the tissues examined (8/12) presented both cell types with positive and negative prostatic staining, the differential expression of prostatic in the BPH glands does not seem related to systemic factors but instead may be indicative of what goes on in the prostate microenvironment. For example, the expression pattern may be determined by whether a particular region of the tissue was more exposed to prostatic expression regulatory factors, such as inflammation. We had previously shown that inflammation is a prostatic expression silencing event in the mouse bladder [23], and induction of SLUG expression by androgen could also repress prostatic expression in the prostate [15]. It is also possible that androgen receptor expression states of the BPH tissues might affect prostatic expression as well. Future studies are needed to address the mechanisms of differential prostatic expression in the BPH tissues.

One of the cellular responses to inflammation, specifically the actions of the inflammatory cytokines, is the production of nitric oxide (NO[•]) by the inducible nitric oxide synthase (iNOS), a mechanism also active in the epithelial cells [29]. NO[•] is highly relevant to BPH and treatment of BPH. The roles of NO[•] and of the constitutively expressed nitric oxide synthases (neuronal/nNOS/NOS1 and endothelial/eNOS/NOS3)

in the major clinical symptoms of BPH, namely LUTS and erectile dysfunction (ED), have been extensively studied [30,31]. Drugs aimed at improving clinical BPH symptoms have been shown to involve NO⁻-related mechanisms [32-36], but mostly concern the nerve tissues and other non-prostate tissues. The prostate epithelial cells were reported to express both nNOS (secretory cells) and eNOS (basal cells) [37]. iNOS has been shown by one study to be expressed in all BPH tissues (n = 10, with or without prostatitis) at the mRNA and the protein levels, while not detected in the normal prostate tissues [37]. The iNOS was localized to the smooth muscle and fibroblast cells in this study [37]. In another study, iNOS mRNA was found to be down-regulated in BPH samples (n = 9) at the mRNA level versus the normal tissues (n = 12), using RNA extracted from frozen tissue sections [38]. No localization information was available from the second study, but the average epithelial contents of the specimens were provided. The BPH tissues had an epithelial content of 56% (range of 40-70%) while the normal tissues' was at 69% (55-85%) [33]. Yet a third study reported epithelial-localized iNOS immunoreactivity or expression in samples from patients with BPH, low- or high-grade prostatic intraepithelial neoplasia (PIN), and prostate carcinoma (n = 20 for each type), with high-grade PIN and cancer showing higher intensities than BPH and low-grade PIN [39]. The prostate epithelial-localized iNOS was seen in both the basal and the secretory cell types [39]. We have shown that the BPH-1 cells, which were characterized to be the secretory epithelial type [21], are capable of an induced iNOS expression with the inducing event being prostasin silencing. The heterogeneity of the reported iNOS expression in BPH probably also reflects the heterogeneity of the BPH tissues studied, but at least we confirm the capability of prostate epithelial cells to express

iNOS in response to prostasin expression changes in vitro, suggesting a potential functional role for both prostasin and iNOS in benign prostate diseases.

Along with iNOS, the expression of ICAM-1 was also induced in association with prostasin silencing. We have previously proposed a protease-dependent cytokine signal modulating role for prostasin to regulate inflammatory gene expression [123]. ICAM-1 is a cytokine-regulated gene in cells of the prostate epithelial origin [40], and its expression change in the BPH-1 cells was consistent with this proposed role for prostasin. As opposed to the up-regulation of iNOS and ICAM-1 associated with prostasin silencing in the BPH-1 cells, the expression of cyclin D1 was down-regulated. To explore a potential mechanism for the cyclin D1 down-regulation, we examined the protein expression of EGFR. Prostasin modulation of EGFR-mediated signaling is accompanied by EGFR cleavage in the extracellular domain [20], or changes of EGFR protein expression [19]. In the BPH-1 cells, we did not observe these trademarks for the previously known mechanisms of EGFR signal modulation by prostasin. A novel molecular mechanism, different from that mediated by EGFR, must be suggested for prostasin's regulatory role in cyclin D1 expression.

The increased iNOS expression at the protein level in prostasin-silenced BPH-1 cells, with the expected boost of NO production could be a mechanism for cyclin D1 down-regulation, as previously reported for the human breast cancer cell line MDA-MB-231 using the NO[•] donor DETA-NONOate (1 mM) [41]. There was no notable change in CDK4, CDK6, CDK2, cyclin D2, cyclin D3, or cyclin E [41]. It is shown that this decrease of cyclin D1 is due to a decrease in cyclin D1 synthesis and not due to its degradation [41]. This is evidenced by the fact that a decrease in cyclin D1 protein

preceded the decrease in cyclin D1 mRNA. It was also shown the the NO[•] donor had no effect on the half-life of cyclin D1 in this breast cancer cell line, therefore ruling out degradation [41]. NO[•] must be regulating cyclin D1 at the translational or posttranslational stage [41]. They also ruled out the possibility of cGMP mediating the effects of NO[•] on cyclin D1 [41]. The cell cycle regulatory protein cyclin D1 is expressed at normal levels in clinical BPH tissues [42], and is positively or negatively responsive to transcriptional regulation in the BPH-1 cell line [43]. It was not unexpected that the cyclin D1 down-regulation is accompanied by a growth inhibition effect on the BPH-1 cells because of cyclin D1's established role in cell cycle regulation, and in the BPH-1 cells [25]. It was unexpected, however, that an event involving prostasin expression change resulted in changes of cell proliferation because we had previously shown that re-expression of prostasin in cancer cell lines did not affect cell growth [15]. One could speculate that the cancer cells no longer possess the cellular pathways to respond properly to prostasin on cell proliferation signals, whereas the pathways are intact in the BPH-1 cells to allow this readout. In addition to its cell cycle regulation role, cyclin D1 has also been shown to regulate cell motility via cytoskeleton remodeling and membrane ruffling [44], while over-expression of cyclin D1 in the BPH-1 cells did also result in increased invasion via increased cell motility [15]. The cyclin D1 down-regulation in the prostasin-silenced BPH-1 cells, along with the increased expression of iNOS and ICAM-1 may be responsible for the phenotypic change to almost completely non-invasive. But this finding seemed to contradict our previous observations that prostasin acted as an invasion suppressor in cancer cells [15]. Again, the new finding that prostasin silencing in the BPH-1 cells actually reduced, instead of increasing cell

invasiveness or motility may be the result of different molecular mechanisms at play. We evaluated the proteolytic profiles of matrix metalloproteases (MMP-2 and MMP-9) by gelatin zymography assays of the secreted MMP's in the culture medium, no difference was observed for the differently treated cells (data not shown). The reduced invasion of the Pro-siRNA treated BPH-1 cells is most likely a change of migratory potential, which can be directly regulated by cyclin D1 [25], iNOS [45], and ICAM-1 [46].

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